

Production of Recombinant Protein Pap31 and Its Application for the Diagnosis of *Bartonella bacilliformis* Infection

A. TAYE,^{a,b} H. CHEN,^{a,b} K. DUNCAN,^{a,b} Z. ZHANG,^{a,b} L. HENDRIX,^c
J. GONZALEZ,^b AND W. CHING^{a,b}

^a*Rickettsial Diseases Department, Naval Medical Research Center,
Silver Spring, Maryland 20910-7500, USA*

^b*Department of Preventive Medicine and Biometrics, Uniformed Services
University of Health Sciences, Bethesda, Maryland, USA*

^c*Texas A & M University System Health Science Center, College Station, Texas, USA*

ABSTRACT: Tropical bartonellosis is a highly fatal epidemic and endemic infectious disease that occurs throughout the communities of the Andes Mountains in South America. The disease is caused by the facultative intracellular bacteria, *Bartonella bacilliformis*. The emergence of bartonellosis in new geographic areas and an increase in the number of reported cases suggest the need for a rapid test for epidemiologic study and investigation of the disease burden. The objective of this research is to develop a rapid serologic diagnostic test using recombinant antigens to overcome the limitations of the current standard IFA technique for laboratory diagnosis. Western blot analysis with patient sera of whole cell lysate separated on a 2D gel identified Pap31 as a dominant antigen. PCR primers were designed according to the sequence of ATCC strain 35685 to amplify the gene coding for Pap31 from a local isolate (HOSP 800-09, Peru). The amplicon was subsequently cloned into pET24a, adding the T7 tag, and expressed in *E. coli*. Patient sera with different IFA titers confirmed the diagnostic band of 31 kDa on a Western blot of SDS-PAGE. The performance of affinity-purified recombinant Pap31 (rPap31) was also evaluated in an ELISA format with 137 patient sera of known IFA titers. The range of ELISA reading from positive sera did not overlap with the range of those from negative sera, suggesting the potential application of rPap31 in both ELISA for high throughput regional hospital settings and in the construction of handheld rapid tests for rural clinical sites.

KEYWORDS: *Bartonella bacilliformis*; Pap31; ELISA; infection

Bartonella bacilliformis, a small (0.2–0.5 × 1.0 µm), motile, pleomorphic coccobacillus, is the etiological agent of Oroya fever for the acute phase and verruga peruana for the chronic phase of Carrión's disease. The disease is endemic in high-altitude regions of the Colombian, Ecuadorian, and Peruvian Andes. The acute phase is char-

Address for correspondence: Wei-Mei Ching, Rickettsial Diseases Department, Infectious Diseases Directorate, Naval Medical Research Center, 503 Robert Grant Avenue, Silver Spring, MD 20910-7500. Voice: 301-319-7438; fax: 301-319-7460.
chingw@nmrc.navy.mil

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acterized by hemolytic anemia, fever, pallor, and transient immunosuppression.¹ The chronic phase is characterized by cutaneous vascular lesions. Without antibiotics, mortality rates of 40–88% have been reported.^{2,3} Early diagnosis of the disease is very important for effectiveness of therapy. Furthermore, despite endemicity in South America, bartonellosis is becoming an increasingly important health issue with the recent rise in reported cases and the emergence into new locations, increasing the potential threat to overseas travelers.⁴

A number of techniques are currently available for the diagnosis of *B. bacilliformis* exposure and infection. However, they all suffer from being nonspecific, extremely laborious, expensive, or time-consuming. These limitations are especially acute when the required tests are conducted by facilities with limited resources. Laboratory culture of *B. bacilliformis* is hampered by the bacterium's fastidious nature. It requires two to five weeks to culture and identify the isolate. Peripheral blood smears and histopathologic studies are the most frequently used methods of diagnosis. However, the sensitivity of this method is only 36% when compared to bacterial isolation.² PCR amplification of various *B. bacilliformis* specific genes has been demonstrated to be useful. While these molecular techniques offer high sensitivity and specificity, PCR assays require equipment and expertise not widely available in endemic areas.

Serological assays using whole-cell antigen or recombinant 65-kDa antigen in ELISA or Western blot have been reported, but suffered from lack of specificity. The current gold standard method, indirect fluorescence assay (IFA), uses irradiated whole-cell antigen preparation from cocultivated Vero cells.⁵ The method is sensitive, but costly, and requires expertise to achieve reproducible results. Because *B. bacilliformis* is the causative agent of Oroya fever and verruga peruana, where mortality can be exceptionally high, improved methods to detect *B. bacilliformis* exposure are needed over existing procedures. We have identified a protein antigen, Pap31, a homologue of a bacteriophage-associated protein in *Bartonella henselae* (also called HbpA), that is an excellent candidate for a diagnostic reagent.⁶ The Pap31 protein, from the virulent Peruvian strain of *B. bacilliformis*, is found to be a highly expressed antigen in growing cultures of *B. bacilliformis*. Furthermore, the protein is immunologically dominant, making it an ideal antigen for use in enzyme-linked immunosorbent assays (ELISA) and in immunoblotting assays. The Pap31 has been sequenced and a recombinant Pap31 can be produced in bulk. The use of this single protein antigen permits the use of ELISA, Western blot methods, or rapid lateral flow assay for the diagnosis of *B. bacilliformis* infection. All these methods are preferable over currently available regimens.

MATERIALS AND METHODS

Identification of Pap31 as the Immunodominant Antigen

Bartonella bacilliformis strain ATCC KC583 was grown on 5% rabbit blood agar plates at 28°C in the absence of CO₂. After 10–14 days, the bacteria were harvested with BHI medium (3.5 mg/mL). Total bacterial proteins (30 µg) were separated via 2D gel electrophoresis. The gel was either stained with SYPRO (Bio-Rad Laboratories, CA) or electrotransferred onto a polyvinylidene difluoride (PVDF) membrane.

Seroreactive protein antigens were detected by immunoblotting with a patient serum. The corresponding 5 protein spots were cut out for N-terminal amino acid sequencing.

Cloning, Expression, and Purification of Recombinant Pap31 (rPap31)

Primers for rPap31 were designed according to the ATCC strain 35685 (KC583) sequence determined by L. Hendrix. The sequence has just been submitted to GenBank (accession number DQ207957) (forward primer: gcagcatatgttatgatcccgaagaaata; reverse primer: ctaaaggcacaaccacaacgcattcttaag). The rPap31 gene segment was amplified using the genomic DNA of a local strain HOSP 800-09 as the template. PCR product was inserted between the NdeI and EcoRI sites of the expression vector pET24a (pET24a-pap31). The rPap31 protein was expressed in *E. coli* BL21 (DE3) after induction with 1 mM IPTG. The Pap31 gene segment in pET24a was recloned

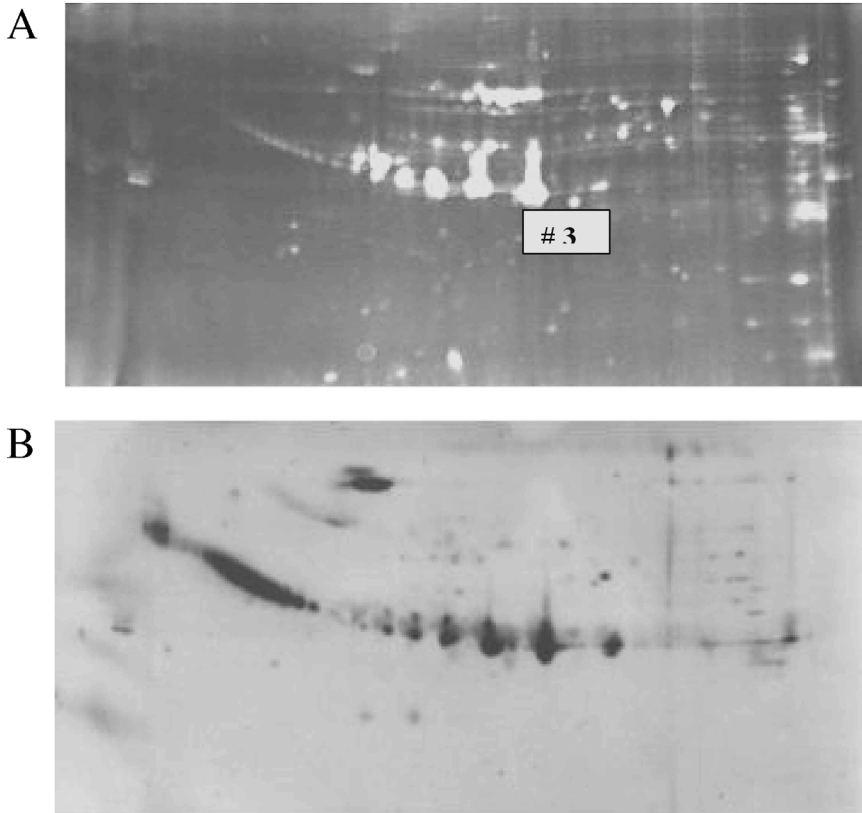


FIGURE 1. Two-dimensional gel electrophoresis and Western blot analysis of proteins from *Bartonella bacilliformis* whole-cell lysate. **(A)** Total protein stained by SYPRO in a pH range of 3–7. **(B)** Western blot analysis of the protein shown in *panel A* staining with serum from a *B. bacilliformis*-infected patient.

by GenWay Biotech Incorporated (San Diego, CA) to attach a T7 tag to the N-terminus of the rPap31 gene insert. The rPap31 was expressed as an inclusion body, which was washed and solubilized with 8 M urea in the presence of 1% Triton X-100 and 2 mM DTT in 50 mM Tris HCl, pH 8.0. The polypeptide was refolded by dialysis against 0.1 mM EDTA/12% glycerol in 50 mM Tris-HCl, pH 8.0, at 4°C. The refolded protein was further purified using the T7-tag affinity column in the presence of 2 M urea.

Evaluation of rPap31 for Serological Reactivity

Sera were collected from Peruvian patients with acute or chronic phase bartonellosis in 2000. Western blot analysis and ELISA were carried out as previously described.⁷

RESULTS AND DISCUSSION

Under 2D gel electrophoresis of the lysed whole-cell total protein, 4 to 5 moieties migrated near, but not identically, to the Pap31 protein (FIG. 1). All spots were cut out of the gel and subjected to amino acid sequencing. Of the 5 dominant proteins, spot no. 3 yielded the longest amino acid sequence, closely resembling that of *B. henselae* Pap31. Western blot analysis with IFA-positive patient sera proved that it was the immunodominant antigen. For the IFA test, sera with titers greater than 128 were designated as positive and those with 128 and below as negative for bartonella infection. For the Western blot, the diagnostic band is at 30.3 kDa, the size of our recombinant protein antigen, rPap31 (FIG. 2). Patient sera were diagnosed as positive if reactive with a band of this size. Diagnosis was confirmed by comparing

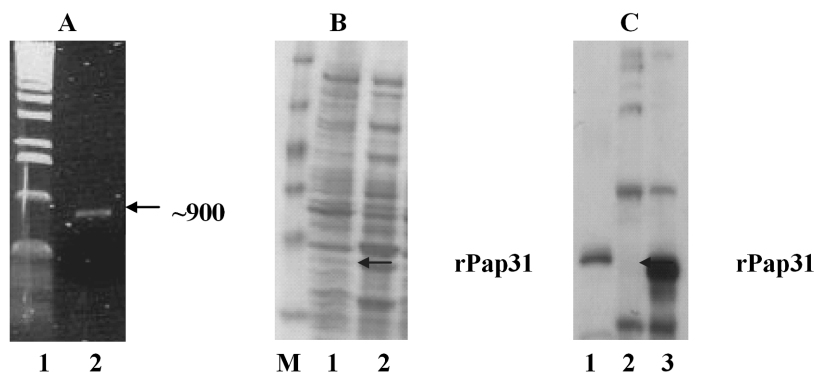


FIGURE 2. Cloning and expression of recombinant Pap31 (rPap31). (A) Amplification of the Pap31 gene by PCR: lane 1, 1-kb ladder; lane 2, PCR product. (B) Expression of the recombinant antigen rPap31 in *E. coli* BL21 (DE3) by vector pET24a-Pap31: lane 1, uninduced; lane 2, induced. (C) Confirmation of the seroreactivity of rPap31 by Western blot analysis with patient serum: lane 1, *B. bacilliformis* cell lysate; lane 2, BL21 (DE3) carrying pET24a; lane 3, BL21 (DE3) carrying pET24a-Pap31.

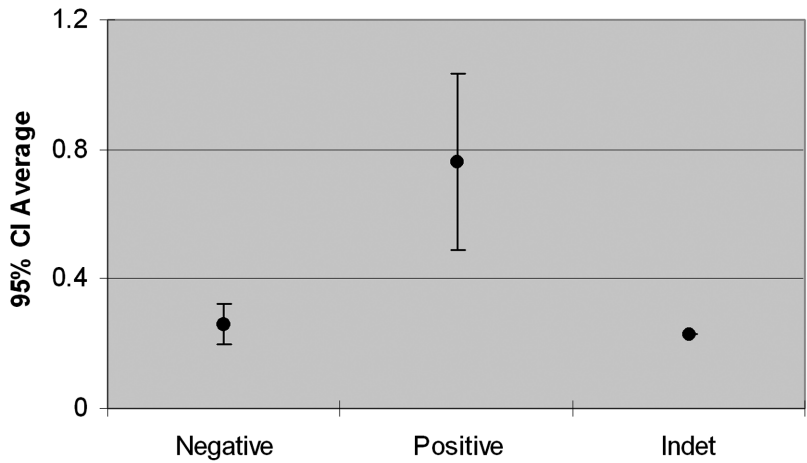


FIGURE 3. ELISA results versus IFA results for 137 sera: mean and 95% CI of ELISA OD by IFA result. Samples with IFA titers equal to or higher than 256 were considered as positive. Indetermined (“Indet”) means that, after repeated testing (5 times) by IFA, the sample cannot be given as a clear positive or a clear negative.

the membrane with those incubated with the negative control serum and the positive control patient serum. The rPap31 exhibited both high sensitivity and specificity in ELISA (Fig. 3). Among the 137 samples tested, 107 were IFA-negative, 29 were IFA-positive, and 1 was indetermined.

The ELISA OD range (95% confidence interval) of the IFA negatives did not overlap with those of the IFA positives. The rPap31 is easy to purify and store, and can go through several freeze-thaw cycles without losing its antigenicity. The protein would be well suited for use in any antibody-based assay, including ELISA, Western blotting, and rapid immunochromatographic assays.

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[*Competing interests statement:* The authors declare that they have no conflicting financial interests.]

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